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Isolation and Partial Characterization of Serogroup Specific Antigens from Bradyrhizobium japonicum 61A123

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Isolation and Partial Characterization of Serogroup Specific Antigens from *Bradyrhizobium japonicum* 61A123

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BY

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I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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Isolation and Partial Characterization of Serogroup Specific Antigens from *Bradyrhizobium japonicum* 61A123

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ABSTRACT

Serogroup specific antigens of Bradyrhizobium japonicum 61A123 were isolated using an immunochemical assay. Bacterial antigens were isolated by phenol-water extraction. These antigens were detected by inhibition of an enzyme linked immunosorbant assay (ELISA). Low pressure chromatography and digestion with proteinase K served to purify the antigens. Chemical composition was determined by gas chromatography (GC), mass spectrometry (MS), and colorimetric assays. The antigens were also characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and immunological properties determined by ELISA. These antigens were shown to be lipopolysaccharides (LPSs) containing fucose, a 2-amino-2,6-dideoxy hexose (unidentified), glucose and mannose as the major sugars, along with small amounts (<1%) of 2-keto-3-deoxyoctonate (KDO). Fatty acid determination showed the presence of 3-hydroxy myristic, lauric, oleic, and two unidentified fatty acids as major components. The growth medium for the bacteria was modified to supress the excessive production of extracellular polysaccharides (EPSs). This medium had no qualitative effect on the nodulation or immunochemical characteristics of the bacteria. Five hours of mild acid hydrolysis (1% acetic acid at 100^{o}C) was required to separate the lipid from the polysaccharide portion of the molecule.

VITA

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INTRODUCTION

Nitrogenous fertilizers today are made mostly from reduction of atmospheric dinitrogen, N_2, by a reaction known as the Haber-Bosch process requiring high pressure, moderately high temperature and a good catalyst. In addition to the practical task of providing the huge supply of nitrogen compounds necessary for industrial and agricultural uses as inexpensively as possible, chemists are attracted by the possibility of discovering processes that will work under less drastic conditions. They do exist! A soybean plant can be "watched" growing at 1 atm and 28°C.

Remarkably, the biological fixation of atmospheric dinitrogen is restricted to the more simple organisms, free-living species of blue-green algae and symbiotic species of bacteria. These include the aerobic genera Azotobacter, Rhizobium, and Bradyrhizobium (27), facultative aerobes like Klebsiella, Enterobacter and Bacillus, and the strictly anaerobic Clostridium. Ecologically, the most important nitrogen-fixing systems are those which 'fix' in association with a plant, because the biologically useful form of nitrogen (i.e. ammonia) is supplied just where it is needed, at the plant roots (37).

This fundamental process is best accomplished by bacteria of the genera Rhizobium and Bradyrhizobium through symbiotic infection of various species of legumes: peas, beans, soybeans, chickpeas, alfalfa, clover, and so on

(37), in a step-wise pathway common to most rhizobia-legume symbioses. These steps are: a) attachment of the bacteria to the root hairs of the host plant; b) curling of the root hairs; c) development of infection threads in the root hairs; d) release of the bacteria from the infection thread into the inner cortical cells of the root; and e) formation of the nitrogen reducing bacteroids (51).

The infection process is highly specific since it proceeds invariably through host-symbiont recognition. For instance, Bradyrhizobium japonicum will infect only soybeans, whereas Rhizobium trifolii will infect only clover, and Rhizobium leguminosarum only peas (37). In addition, within the same species there are a number of strains that have the ability to infect the same host, but with different efficacy. That is, one strain will out-compete the others for nodule occupation. The basis for this "competition" among Rhizobium and Bradyrhizobium strains for their legume host has not been determined, yet it is a complex process known to involve symbiont and host genes in addition to numerous soil factors such as pH, soil type, moisture, salinity, and temperature (15,21,26,33,38).

Within Bradyrhizobium japonicum strains, serogroup 123 is particularly abundant in midwestern soils and is highly competitive in the infection and nodulation of soybean plants. Unfortunately it is not a very efficient nitrogen fixing serogroup. Attempts to control the competitiveness of this indigenous strain by introducing

large amounts of a desired inoculum in the vicinity of the root, and thus hoping to out-compete the indigenous strain, has had minimal effects on nodule occupancy (53,54). In fact, even under these conditions, serogroup 123 will occupy from 50% to 100% of soybean nodules (32). Presently, the use of naturally occurring viruses, known as bacteriophages, which have the ability to kill the indigenous strain(s) but not the inoculum strain, is under investigation to promote the establishment of superior inoculum strains (3,16,47).

Given the importance of species specific interactions as an agricultural tool, and in order to further understand the biochemistry of competition, the effort of this work has been centered on characterizing the molecules thought to be responsible for establishing an effective symbiosis between Bradyrhizobium japonicum strain 61A123, and its legume host, soybean (Glycine max).

While there are a number of reports which suggest that surface polysaccharides, extracellular, capsular and lipopolysaccharides (EPSs, CPSs, and LPSs) from Rhizobium and Bradyrhizobium are of major importance in the establishment of an effective symbiosis with the host legume (4,6,13,20), most of the work has been concentrated on the structure and chemical composition of EPSs and CPSs. In contrast, there are only few reports in the literature that describe the partial compositions of LPSs isolated from Rhizobium species (6), and only one published report on the composition of LPS from the slow-growing

Bradyrhizobium (44).

The importance of Rhizobium and Bradyrhizobium LPSs can be explained by comparison to other Gram-negative bacteria. For instance, it is well known that LPSs of pathogenic bacteria are involved in the recognition between these bacteria and their potential hosts (48). The structure, synthesis and biological properties of these LPSs have been extensively discussed (2,25). Since LPSs are common components of all Gram-negative bacteria, it is likely that LPSs from symbiotic bacteria are also involved in the recognition of potential hosts (e.g., legumes). In the same way, biological properties may be similar for LPSs of pathogenic and symbiotic bacteria.

There are a number of reports which suggest that the LPSs from Rhizobium and Bradyrhizobium play an important role in the symbiotic infection of legumes. Studies on the early stages of infection have indicated that LPS from Rhizobium binds specifically to root proteins called lectins (28,29,1). Whether this is an indication of specificity in attachment has not been clarified. In fact several subsequent reports show that there is no correlation between attachment and the specificity of symbiosis (43,58). There are supportive data, though, that LPSs may be important in later events of symbiosis. For instance, a recent report showed that purified LPS from Rhizobium trifolii enhanced infection thread formation in clover root hairs (14). Other reports suggest that LPSs from

Rhizobium leguminosarum can inhibit the binding of this bacterium to its legume host (28,29). In addition, it has been reported that the LPSs from bacteroids (the form of rhizobia found inside the nodules) differ from those of free-living (cultured) bacteria, in that the number of glycosyl residues found in the O-antigen side chain have been significantly reduced (42,50). This alteration may be necessary for the exchange of metabolites between the host plant and bacteroid (Carlson, unpublished data). Two reports show that a symbiotic defect is linked directly to a defect in the LPS. In one of them, Bradyrhizobium japonicum HS123, a nonnodulating mutant of USDA 1110 that does not cause root hair curling, has been shown to have an LPS which is missing the O-chain. The EPS and CPS, however, are the same (44). The second report, in which a Tn5 insertion mutant of Rhizobium phaseoli was used, also shows the absence of the O-chain resulting in the inability to develop an infection thread. Thus, there seems to be significant evidence supporting the role of Rhizobium LPSs in the infection and nodulation of legumes.

Structurally, LPSs are complex molecules that can vary significantly even among strains of a single species (6,9,59). Rhizobium and Bradyrhizobium LPSs are similar to the LPSs of other Gram-negative bacteria in that they usually contain three structural regions, lipid-A, responsible for the toxicity of some Gram-negative bacteria; a repeating oligosaccharide known as the O-antigen, normally

associated with the antigenicity of the bacteria; and a core oligosaccharide (57). In addition, an incomplete form of the LPS, which lacks the O-chain, has also been found in the surface of Rhizobium (7). Generally, the O-antigen (if present) is attached to the core oligosaccharide and the core is attached to the lipid-A through an acid-labile 2-keto-3-deoxyoctanoic (KDO) acid linkage (9,59) as shown in Figure 1. Thus, cleavage of the KDO bond by mild acid hydrolysis (6,9,59) separates lipid-A from the O-chain-core polysaccharide in the complete LPS, and from the core oligosaccharide in the incomplete LPS (57). In some Rhizobium LPSs, however, the arrangement of the polysaccharide region appears to differ from the above description since mild acid hydrolysis releases an O-chain and a core oligosaccharide (7). It has been shown that the O-chain as well as the core oligosaccharide from Rhizobium trifolii have KDO at their reducing end (10). There are results suggesting that this is also the case for Rhizobium leguminosarum and Rhizobium phaseoli LPSs (Carlson, unpublished data). Differences in composition also exist between Rhizobium LPSs and those from other Gram-negative bacteria. For instance, Rhizobium LPSs unlike Salmonella LPSs contain uronic acid and are usually devoid of heptose (9,59).

Purification of LPSs from Gram-negative bacteria has been generally done by the method of Westphal and Jann (56) using 45% hot phenol/water. Rapid cooling of this mixture

results in separation of the water from the phenol phase. In most cases the LPS will be found in the water layer (9), although there are reports of LPSs being isolated from the phenol layer (40,19). The purification of Rhizobium LPS has been followed by assaying for KDO, which has been reported to be present in all of the Rhizobium LPSs examined to date. However, attempts to purify LPSs from several strains of Bradyrhizobium japonicum by using this method, has proven ineffective (8).

This work presents an alternative approach to the isolation and purification of Bradyrhizobium japonicum 61A123 LPSs. It is known that the LPSs of Gram-negative bacteria, including rhizobia, are the major strain specific heat stable antigens of these microorganisms (52). Consequently, rabbit antisera to strain 61A123 was obtained and used in an enzyme linked immunosorbant assay (ELISA) (18) to "follow" the immunodominant molecule as a function of purification. Using this method the LPS from strain 61A123 was isolated and partially characterized.

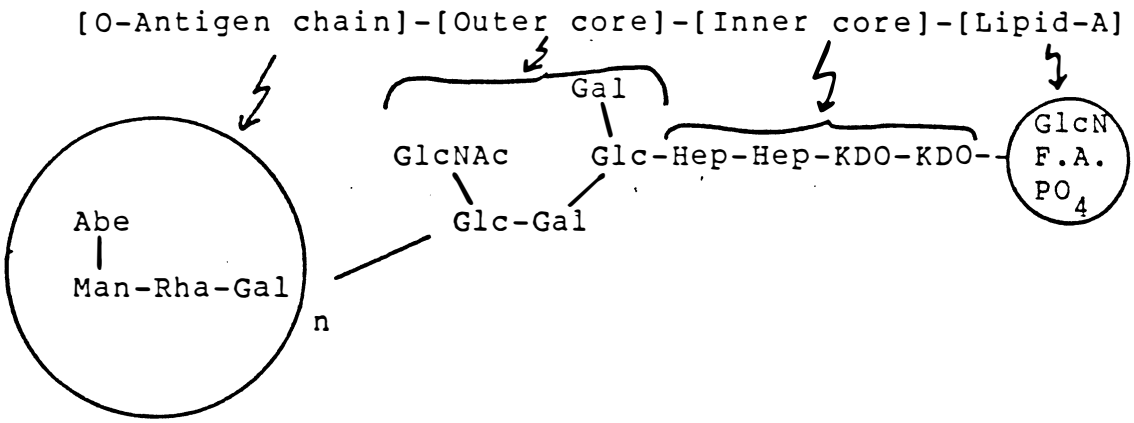

Figure 1. The general structural arrangement of the LPS components for S. typhimurium. Abe, abequose; Ea, ethanolamine; F.A., fatty acid; P, phosphate.

MATERIALS AND METHODS

Organisms

Bradyrhizobium japonicum strain 61A123 was obtained from Wolfang D. Bauer, Charles Kettering Reserch Center, Yellow Springs, OH (Presently at Ohio State University). The bacteria were maintained on solid yeast extract-mannitol (YEM) medium [Appendix IA] and stored at 4°C.

Growth Conditions

Bacteria were transferred to 125 mL flasks containing 40 mL of sterile YEM or new yeast extract glutamate (YEG) medium [Appendix IB], and grown at room temperature on a shaker, for proper aeration, at 150 rpm to early stationary phase (about one week). These cultures were used to inoculate 500 mL of sterile medium in one liter flasks and grown as above to mid-log phase. Growth was monitored by measuring the optical density at 620 nm. The bacteria were harvested by centrifugation, in a Sorvall SS-3 or SS-4 centrifuge using a GSA rotor, at 9,000 rpm for 15 minutes at 4°C. The cell pellet was collected and frozen (-20°C) until ready to use. Cultures were checked for Gram reaction, antibiotic resistance (500 ug/mL spectinomycin), and inability to grow in nutrient agar before inoculation of the one liter flask and before harvesting to recognize contaminants.

Enzyme Linked Immunosorbant Assay (ELISA)

Enzyme linked immunosorbant assay procedure (18,10) was

used to "follow" antigenic molecules as a function of purification and it is described in Appendix V. In this assay a known antigen, in this case Bradyrhizobium japonicum 61A123, is adsorbed to a solid support such as the well of a polystyrene microtiter plate. Rabbit antisera to this antigen will bind to the well and it can be detected by incubation with anti-rabbit goat antisera that is linked to an enzyme such as alkaline phosphatase. The presence of this enzyme is observed by addition of para-nitrophenyl phosphate which is hydrolysed to para-nitrophenol which in turn is yellow in a basic solution. The absorbance of para-nitrophenol can be measured at 405 nm. Molecules that bind to the antisera will inhibit its binding to the antigen on the solid support and the yellow coloration will not be observed. Thus, purification of antigenic molecules from the bacteria membrane can be followed by this inhibition assay.

Antigen Extraction

Antigens were extracted by the phenol/water method (56) as modified by Carlson et al.(9) [Appendix II]. Both layers were dialyzed against deionized water and lyophilized. The dry phenol layer was suspended in water, shaken vigorously and centrifuged at 7,000 rpm for 15 min. The supernatant was carefully decanted and saved. This process was repeated three times and the supernatants were combined and lyophilized. The resulting water-soluble phenol fraction (WS) corresponds to approximately 10% of the total mass of the dried phenol layer. The rest of the material,

water-insoluble phenol fraction (WI), was also lyophilized.

Antigens were also extracted by the EDTA/chloroform/methanol procedure (40) [Appendix XIV], in which EDTA antigen preparations (36) were subjected to Folch extraction (17) for further purification.

Antigen Purification

The water soluble material was purified by gel filtration chromatography using a 112 x 1.0 cm column of Sepharose 4-B or Sephadex G-50 in PBS-tween buffer at pH 7.4 [Appendix V]. Fractions collected (150 x 1.0 ml) were assayed for their ability to inhibit the ELISA, as described above, and for hexose [Appendix III] (see Figure 5). Fractions giving 50% inhibition and higher were pooled, dialysed extensively against deionized water and lyophilized. The antigens were further purified by treatment with proteinase K (PK) for protein digestion. To a 10 mg/mL solution of antigenic material in PBS-tween buffer pH 7.4, 1 mg of PK was added (10:1 ratio) followed by incubation at 37^{o}C for 24 hours. Control samples without PK were incubated likewise. All samples were extensively dialyzed against deionized water and lyophilized.

Polyacrylamide Gel Electrophoresis (PAGE)

Discontinuous slab gel electrophoresis with sodium dodecyl sulfate (SDS) was performed by the method of Laemmli (35) (see Appendix VI). The separating gel contained 15% acrylamide, and the stacking gel contained 5% acrylamide. The gels and running buffer contained 0.1% SDS, and the

sample buffer contained 2% SDS. The samples were electrophoresed at a constant current of 20 mA, and the gels were silver-stained as described by Hitchcock and Brown (24) (see Appendix XV).

Immunoblot Procedure

Immediately after running a slab gel, as described above, the samples were electroblotted onto nitrocellulose paper using a Hoeffer Transphor unit. The voltage was set at 80 V for 1 hr. The transfer buffer was 25 mM Tris, and 187 mM glycine prepared in a 20% methanol solution. The nitrocellulose paper was stained for Bradyrhizobium japonicum 61A123 antigens using a modification of a previous procedure (12). The paper was placed in 100 mL of TBS buffer (50 mM Tris, 200 mM NaCl, pH 7.4/HCl) containing 0.5% Knox gelatine. After 30 min, 1 mL of Bradyrhizobium japonicum 61A123 antisera was added to the solution and this was allowed to shake slowly for 2.5 hrs. The antiserum solution was removed, replaced with TBS, and allowed to shake for 10 min. This was repeated two more times. After the final rinsing, the solution was removed and 100 mL of TBS containing 0.5% Knox gelatine and 0.1 mL of peroxidase conjugated antirabbit goat antisera (Sigma Chemicals) was added. This was allowed to shake slowly overnight at room temperature. The nitrocellulose paper was rinsed with TBS as above and then developed in a solution containing 10 mL of 3 mg/mL 4-chloro-1-naphthol in methanol, 40 mL of TBS and 20 uL of 30% hydrogen peroxide (12). The paper was removed,

rinsed in deionized water and allowed to dry.

Composition Analysis

The hexose composition of the antigens was determined qualitatively and quantitatively by gas chromatography (GC) (1) of the alditol acetate derivatives on a Hewlett-Packard Model 5890A gas chromatograph, equipped with a 0.2 um df by 15 m Fused Silica Capillary SP-2330 column (Supelco) and a flame ionization detector (FID). The antigens were hydrolysed with 2 N trifluoroacetic acid, reduced with sodium borohydride to the alditols, and acetylated by heating with acetic acid in pyridine to obtain the alditol acetate derivatives [Appendix XII]. Identification was done by comparing retention times to authentic standards. Identification of hexoses for which standards were not available was accomplished by using combined GC-mass spectrometry at the NIH GC/MS facility at Washington University, St. Louis, MO. The amount of each sugar was determined by integration of the area under the peaks with a computer data system (Chromatochart by IMI, State College, PA). These amounts were corrected for differences in the FID response using a correction factor. This was calculated from the ratio of the area of a known amount of a standard to a known amount of inositol, the internal standard of all samples. Uronic acids were quantitated by the method of Blumenkrantz and Asboe-Hansen (5) using glucuronic acid as a standard [Appendix IX]. KDO, acyl groups, and pyrurate were also determined by colorimetric assays (55,23,30) [Appendixes IV, X, and XI, respectively] using authentic

KDO, glucose pentaacetate, and pyrurate as standards, respectively.

Fatty acids were identified and quantitated by slightly modifying a previously described procedure (45) [Appendix XIII]. Antigen preparations, 500 ug, were mixed with 100 ug of lauric acid, used as internal standard, and hydrolysed in 4 N HCl for 2 hours at 100°C, followed by alkaline hydrolysis with 4 N NaOH for 2 hours at 100°C, acidified with HCl and extracted three times with petroleum ether. The ether layers were kept at 4°C and evaporated to dryness with filtered air. Methyl esters of the fatty acids were prepared by adding 500 uL of BF_3/methanol, heating at 100°C for 2 min., cooling on ice and extracting with petroleum ether. The ether layers were evaporated as above. The resulting samples were dissolved in dichloromethane and analyzed by GC on a SPBl 30 meter capillary column (from Supelco) using a temperature program of 150° to 250°C at 4°C/minute. Identification of fatty acids was made by comparing retention times to a standard mix obtained from Supelco.

RESULTS

Growth Medium

During early stages of this work bacteria were grown on yeast extract-mannitol medium, described in Appendix IA. However, it was very difficult to remove the bacteria from suspension due to the large amount of extracellular and capsular polysaccharides (EPSs and CPSs) produced, in fact even at 27,000 x g only a loose pellet was obtained. To harvest the bacteria it was necessary to make the medium 1 M in NaCl and homogenize it in a blender. Yet this was not sufficient to prevent interference of these molecules in the purification of antigens. Consequently the growth medium was modified to suppress EPS and CPS production. This medium is similar to that reported by R.E. Tulley at Louisiana State University (49). Preparation of the new medium is described in Appendix IB. Although bacterial growth is slower (Figure 2a), the antigenic properties as determined by ELISA remain virtually unchanged (Figure 2b). In addition, nodules formed in soybean roots inoculated with bacteria grown in either regular (YEM) or modified (YEG) media, were qualitatively identical.

Unless specifically indicated, all results in this thesis were obtained with bacteria grown in YEG medium.

Enzyme Linked Immunosorbant Assay

This sensitive test was used to track the antigens as a function of purification. Fractions from every purification

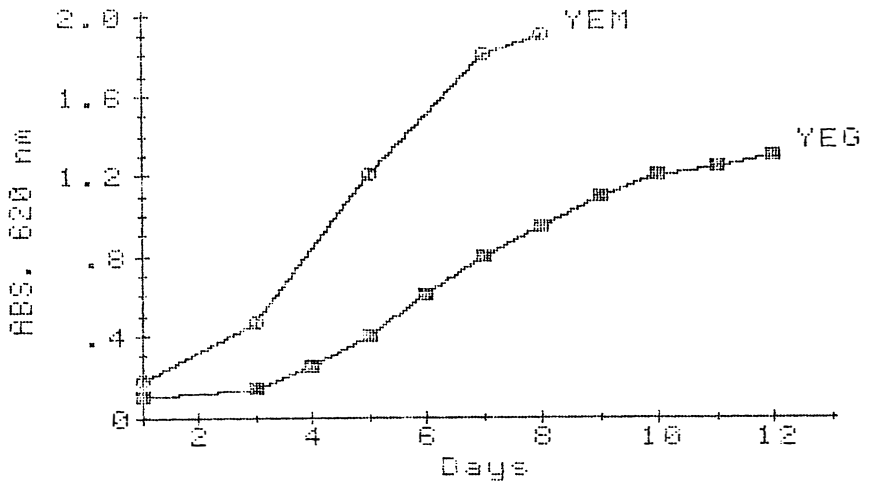

Figure 2a. Growth curves of Bradyrhizobium japonicum 61A123 in yeast extract-mannitol (YEM) and yeast extract-glutamate (YEG) media.

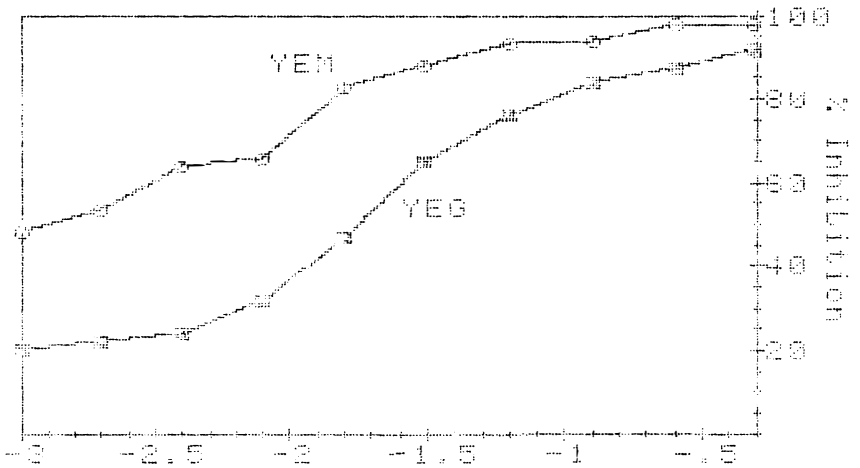

Figure 2b. Inhibition of the ELISA by B. japonicum 61A123 grown in YEM or YEG media. For the x axis, O.D. 620 nm refers to the optical density of the bacterial culture measured at 620 nm.

step were assayed to determine their ability to inhibit the ELISA. Percent inhibition was determined as follows:

$$\frac{A_{405}Bj - A_{405}AM}{A_{405}Bj} \times 100 = \% \text{ inhibition}$$

Where:

A_{405} = Absorbance at 405 nm

Bj = known antigen, in this case Bradyrhizobium japonicum 61A123

AM = any antigenic molecule that will bind to Bradyrhizobium japonicum 61A123 antisera, thus inhibiting its binding to the known antigen, Bj

Extraction and purification of Antigens

When cells grown in YEM or YEG media were extracted by the phenol/water method of Westphal and Jann (56) as modified by Carlson et al.(9) no antigenic activity was detected in the water phase, which would be expected to contain the lipopolysaccharide. The phenol phase, however, yielded an antigenic extract with an activity similar to that of the whole cell (Figure 3). These results suggest the possible presence of a hydrophobic lipopolysaccharide as observed recently in another Gram-negative bacterium, Legionella pneumophila (40).

Suspension of the lyophilized phenol phase in water yields water-soluble (WS) and water insoluble (WI) antigenic fractions that inhibit the ELISA 72% and 57% respectively, at a concentration of 250 ug/mL. Further purification of

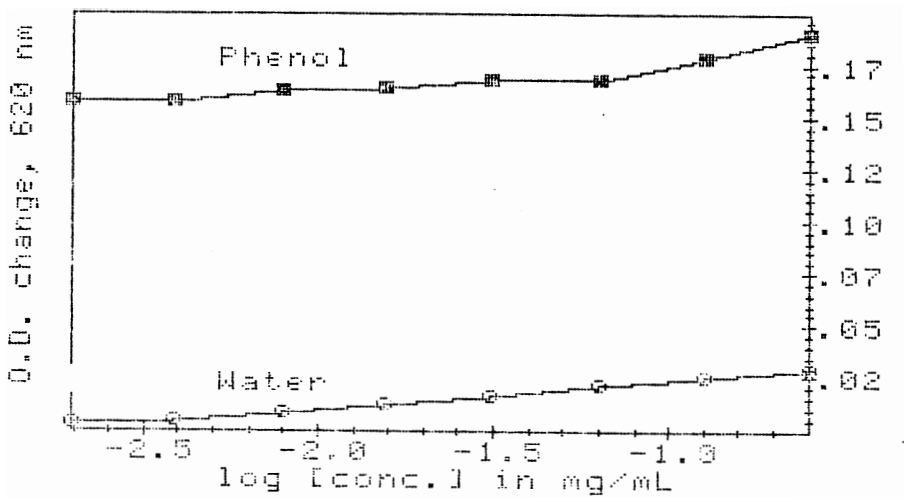

Figure 3. ELISA of the material isolated in the water and the phenol layers after extraction of B. japonicum by the hot phenol/water method (56). This graph represents a direct ELISA in which the material isolated from either layer was directly bound to a microtiter plate. This is not an inhibition assay.

these antigens by digestion with proteinase K (PK) in PBS-tween buffer pH 7.4, at 37°C for 24 hours increased their antigenic activity significantly, as determined by inhibition of the ELISA, 97% and 87% for WS+PK and WI+PK fractions respectively. Remarkably, incubation alone, without PK, also increased inhibition to 94% and 91% for WS and WI fractions, respectively (Figure 4). As a result, specific activitites were calculated for all four fractions to detect where the major portion of this antigenic material was found (see Table 1). The major antigen was found in the water soluble fraction. For details see legend for Table 1.

Gel filtration chromatography of the water soluble fraction in a column of Sephadex G-50 or Sepharose 4-B (exclusion limit $3x10^4$ and $5x10^6$ MW, respectively), both in PBS-tween buffer at pH 7.4, results in a single broad peak that elutes shortly after the void volume as detected by ELISA. Hexose assay [Appendix III] of the same fractions, however, results in two overlaping peaks (see Figure 5). These were dialysed and lyophilized independently as O-1 and O-2.

Antigens were also extracted by the EDTA/chloroform/methanol procedure [Appendix XIV] as described in a recent report (40), in which EDTA antigen preparations (36) are subjected to Folch extraction (17) for further purification. This method, however, results in a split antigenicity between the Folch upper layer ($CHCl_3$-methanol-PBS, 3:48:47) and the Folch lower layer ($CHCl_3$-methanol-PBS, 86:14:1). See figure 6. This procedure was not pursued

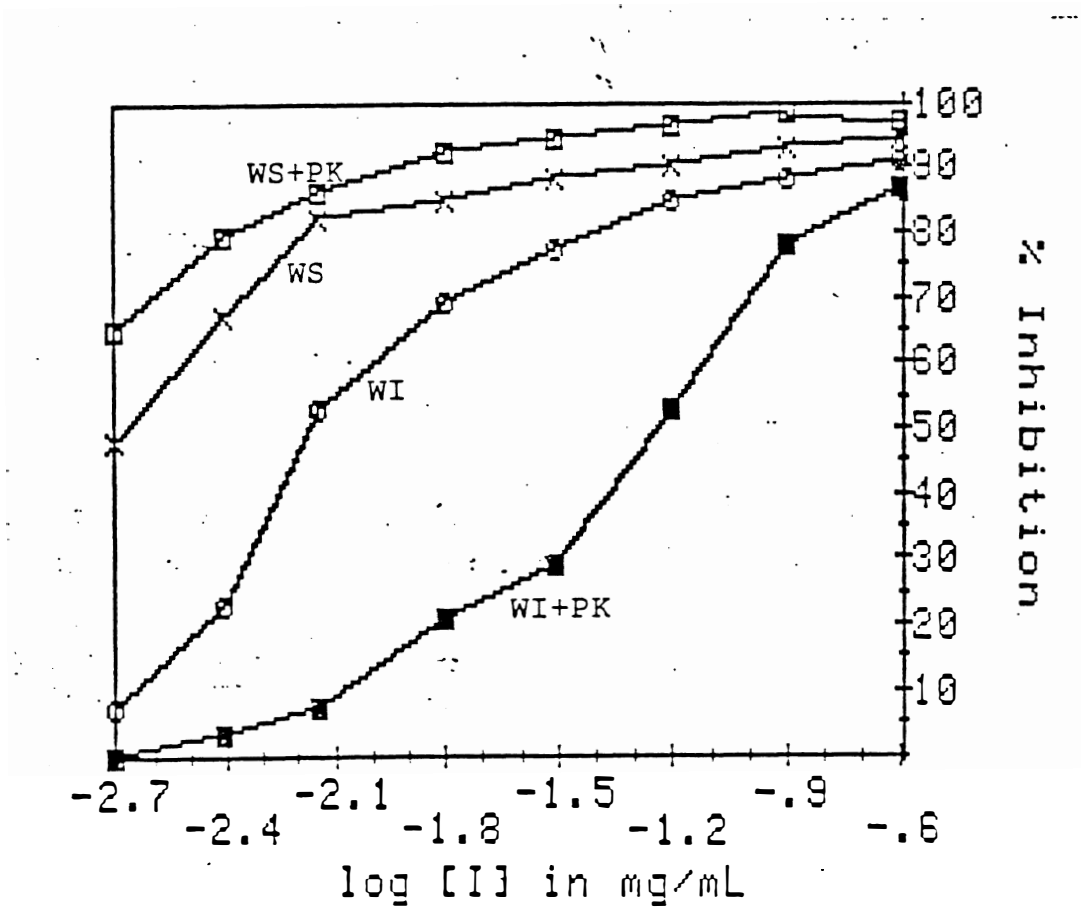

Figure 4. Inhibition of ELISA by the different phenol fractions after digestion with proteinase K for 24 hours at 37°C. Data for the undigested controls is also shown.
WS=water soluble, undigested fraction
WI=water insoluble fraction
PK=proteinase K

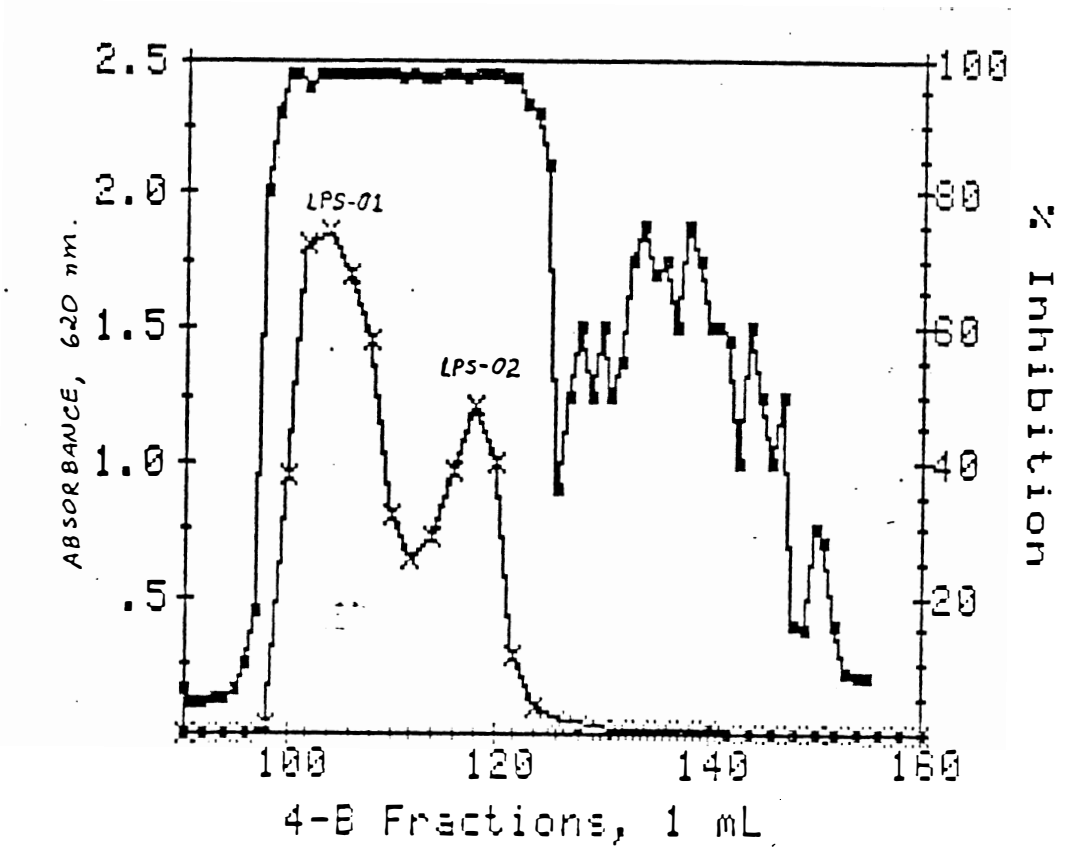

Figure 5. Elution profile of the water soluble (WS) antigens isolated from the phenol phase after hot phenol/water extraction of B. japonicum 61A123. Squares = ELISA, crosses = hexose assay.

Table 1. Inhibitory Activities of Phenol Fractions

Fraction	1 50% inhibition per mg of frac	2 mg per fraction	3 inhibitory activity	4 Relative %
WI	6.60E3	2700	1.8E7	63
WI+PK	869	300	2.6E5	0.91
WS	2.45E4	300	5.4E6	19
WS+PK	4.90E4	100	4.9E6	17

WI Water insoluble fraction
WS Water soluble fraction
PK Proteinase K

Values for column 1 were obtained from a quantitative ELISA assay as that shown in Figure 4.
Values for column 3 were obtained by multiplying column 1 by column 2 .

further.

Attempts to isolate antigens from bacteria grown in YEM medium obtaining first the cell membrane [Appendix VII], after a method described by Schnaitman (46) in which freezing and thawing is used to render Gram-negative cells sensitive to lysozyme or detergents, followed by fractionation into inner and outer membranes using sucrose gradient ultracentrifugation (39), also results in total antigenic activity divided between these two membranes (Figure 7). The major problem associated with this method was the inability to separate the inner and outer membranes due to smearing of the bands, presumably caused by the presence of EPSs. Besides, the cell membrane was irreversibly bound to a DEAE anion-exchange column when chromatographed with a 0 to 1 M sodium chloride gradient in a 0.01 M Tris, 1% Triton-X 100 buffer. This procedure was not pursued further, however the results showed that the major antigens were in the cell envelope.

SDS-PAGE

Bradyrhizobium japonicum 61A123 antigens were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, SDS-PAGE, using the method of Laemmli (35). The silver staining procedure used to visualize the antigens was that of Hitchcock and Brown (24) [Appendix XV]. Both O-1 and O-2 displayed a typical LPS pattern, with three fast-migrating lipid-A-core oligosaccharide bands, a small number of well resolved intermediate migrating bands and

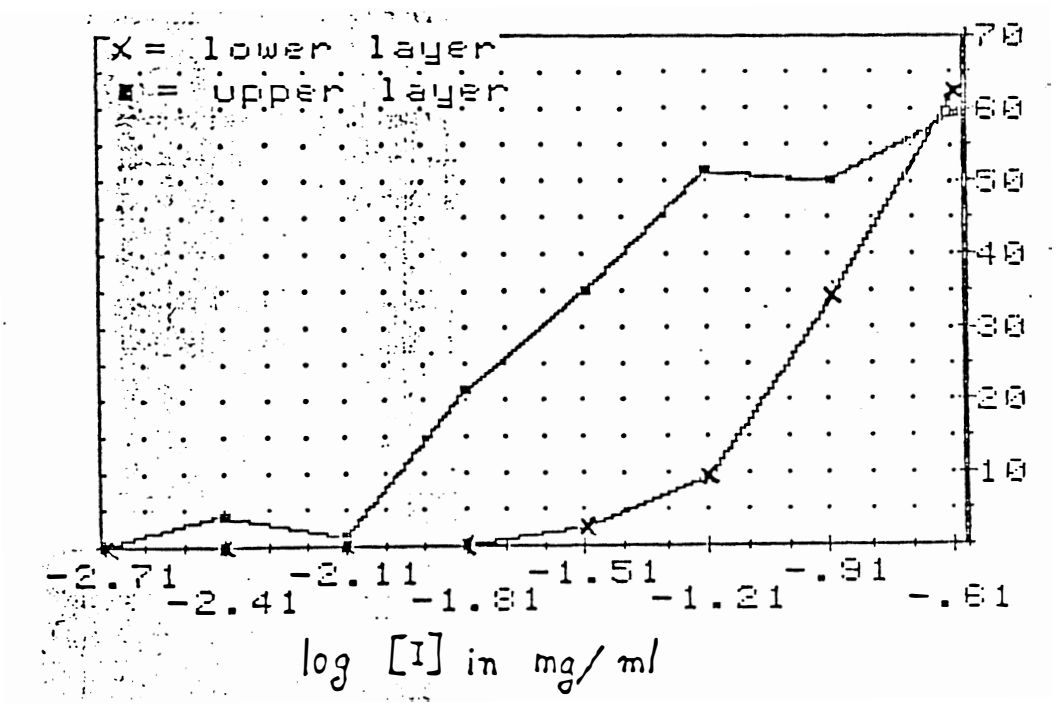

Figure 6. Inhibition of ELISA by antigens of B. japonicum 61A123 extracted by the EDTA-chloroform-methanol procedure.

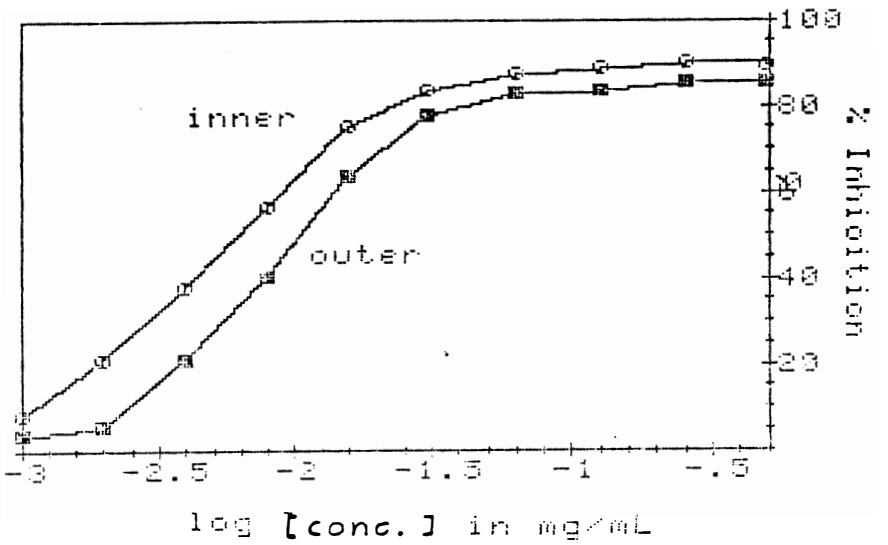

Figure 7. Inhibition of ELISA by the outer and inner membranes of B. japonicum 61A123

several slow migrating bands (Figure 8A). These antigens were also subjected to SDS-PAGE with five-fold increase in detergent concentration of the gels (0.1% to 0.5%) to determine whether the multiple slow migrating bands and the intermediate migrating bands resulted from incomplete dissociation of aggregates. It has been shown that increasing the SDS concentration dissociates the larger aggregation states of Salmonella and E.coli LPSs (41). This modification reduced the number of bands in the upper region (Figure 9), suggesting that incresing the percent of SDS partially disaggregated the LPS. The molecular weight range as compared to protein standards (bovine serum albumin, 66K; egg albumin, 45K; B-lactoglobulin, 18.4K; and lysozyme, 14.3K) is approximately 10-12K for the lower bands, 29-36K for the intermediate bands (increasing regularly by about 1K), 54K for the major upper band and 71-89K for fainter bands of higher molecular weight. These values are given only for comparative purposes and do not necessarily represent the actual molecular weight of the polysaccharides.

SDS-PAGE studies of several other Bradyrhizobium japonicum strains, 61A76, 61A135, 61A101C and mutants ML125, ML126, and ML144 show that their LPSs are also isolated in the phenol phase after phenol-water extraction (Figure 10b). Strain USDA 110 and its nonnodulating mutant HS123, recently analyzed by Stacey's group (44), Rhizobium fredii USDA 205 (previously fast-growing R.japonicum) (11) and Rhizobium leguminosarum ANU 54, all of which

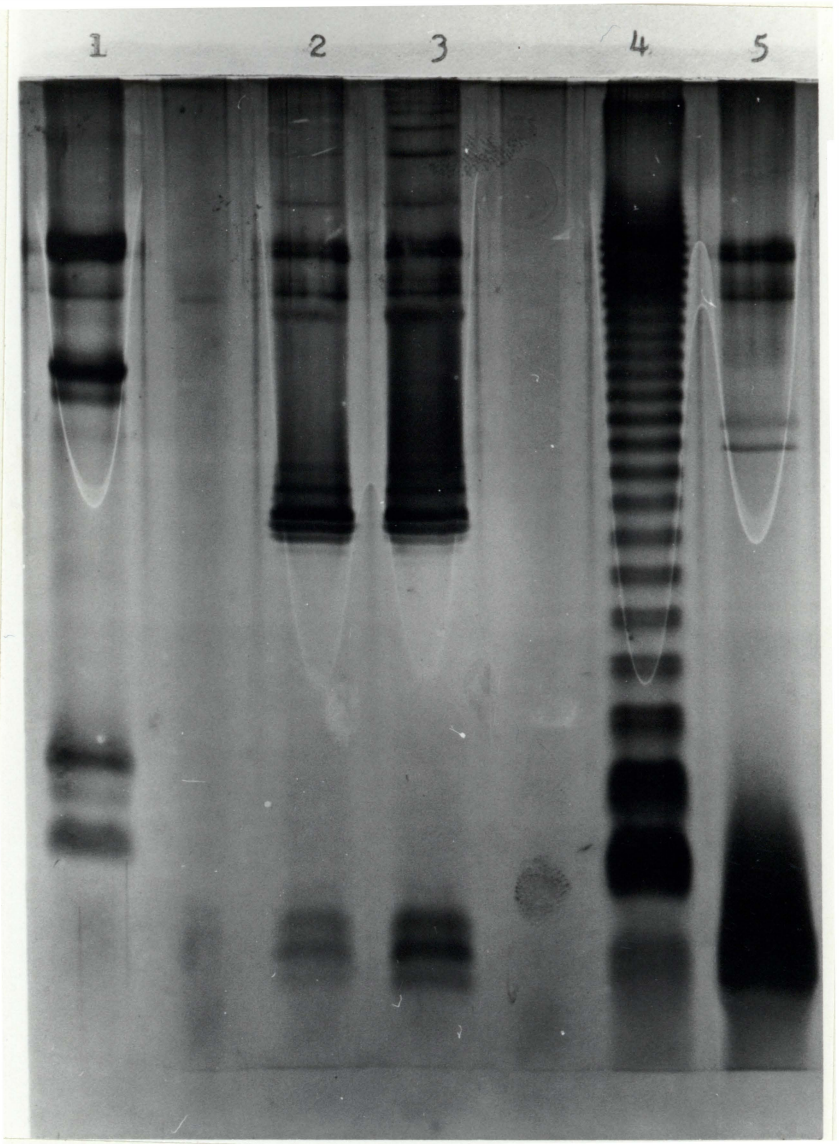

Figure 8a. 0.1% SDS-PAGE. Lane 1: protein standards (BSA, 66K; egg albumin, 45K; B-lactoglobulin, 18.4K; lysozyme, 14.3K), 2 ug of each. Lane 2: LPS O-1, 10 ug. Lane 3: LPS O-2, 10 ug. Lanes 2 and 3 are from B. japonicum 61A123. Lanes 4 and 5 are contain LPS from Salmonella minnesota, wild type and a rough LPS mutant, respectively

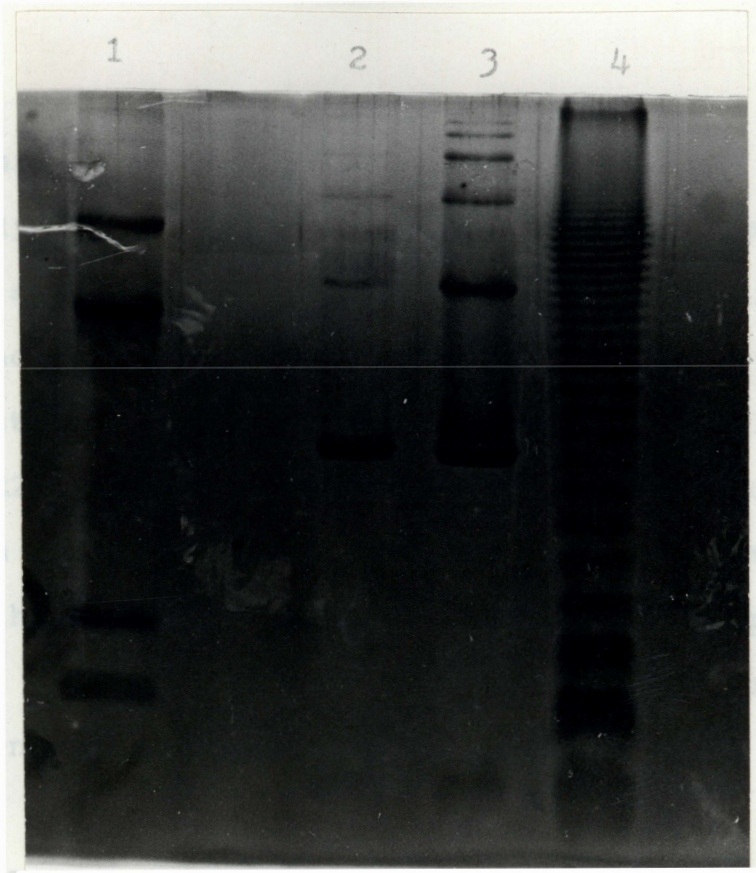

Figure 9. 0.5% SDS-PAGE. Lane 1, protein standards (2 ug of each). For description of standards see figure 8a. Lane 2 and lane 3, B. japonicum 61A123 LPS O-1 and LPS O-2 respectively (10 ug each). Lane 4, LPS of Salmonella minnesota wild type (3 ug).

contain LPS that are isolated in the aqueous phase, were used as comparison. Interestingly, our studies show that LPSs from strains 110 and HS123 are also present in the phenol phase (Figure 10b). These results suggest that the possible presence of hydrophobic lipopolysaccharides may be a general characteristic for Bradyrhizobium japonicum strains.

Immunochemical Analysis of Antigens

SDS-Polyacrylamide gels were subjected to immunoblotting as soon as electrophoresis was complete to determine the antigenic activity of the bands observed in silver-stained gels. Reaction with Bradyrhizobium japonicum 61A123 antisera (22) indicates that only the higher molecular weight form of the LPSs contain the immunodominant site, as judged by the selective staining of this region upon development of the nitrocellulose paper with peroxidase and 4-chloro-1-naphtol (Figure 8b).

Chemical Composition

The LPS compositions are given in Table 2. KDO, acyl groups, pyruvate, and uronic acids were determined colorimetrically as previously indicated. The two major sugars in O-1 and O-2, as determined by gas chromatographic analysis of their alditol acetate derivatives, are fucose, and a 2-amino-2,6-dideoxyhexose which appears after the retention time for standard heptose. Both LPSs contain large amounts of acyl groups and are devoid of

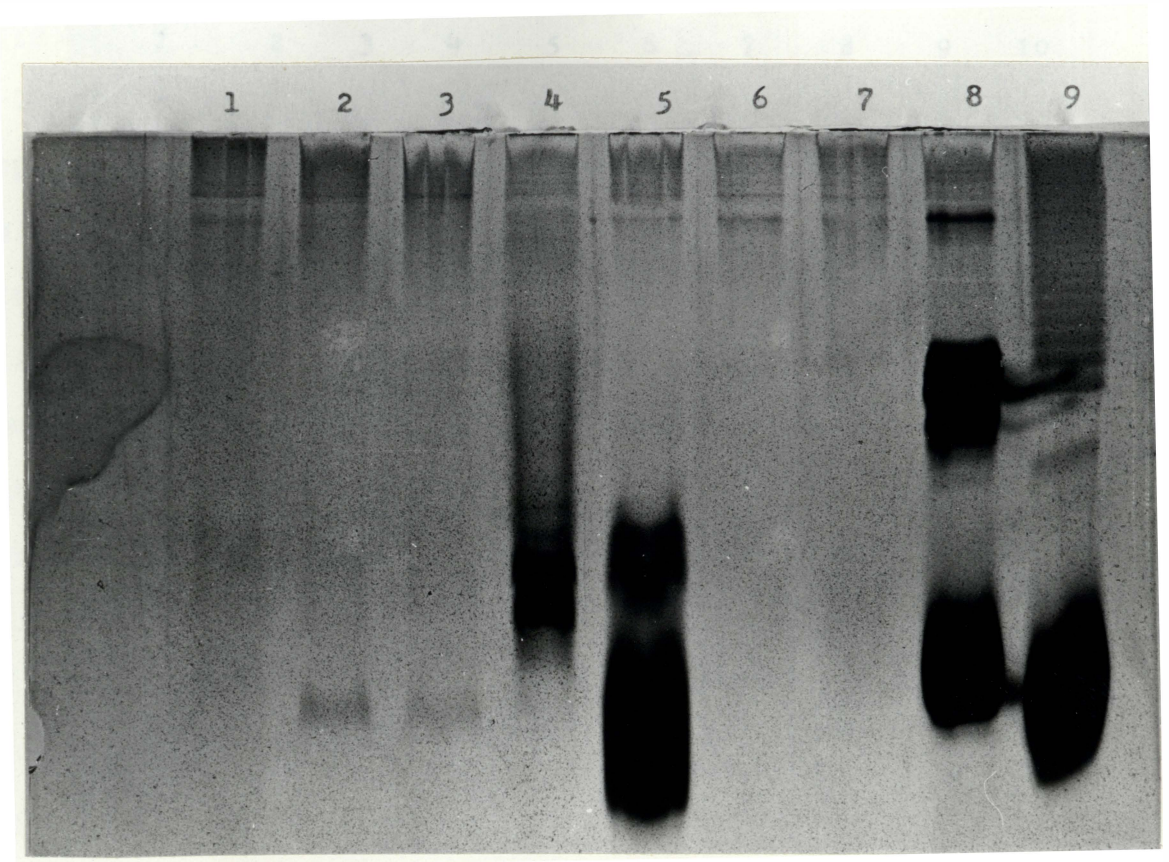

Figure 10a. SDS-PAGE of the material isolated in the water layer after hot phenol/water extraction of several strains of Rhizobium and Bradyrhizobium bacteria. All lanes contain 10 uL of 10 mg/mL solutions. Lanes 1-7 are different strains of B. japonicum: 1, 61A76; 2, 61A101C; 3, ML144, a mutant of 61A101C; 4, USDA 110; 5, HS123, a mutant of USDA 110; 6, 61A123; 7, 61A135; 8, Rhizobium fredii USDA 205; 9, Rhizobium leguminosarum ANU 54.

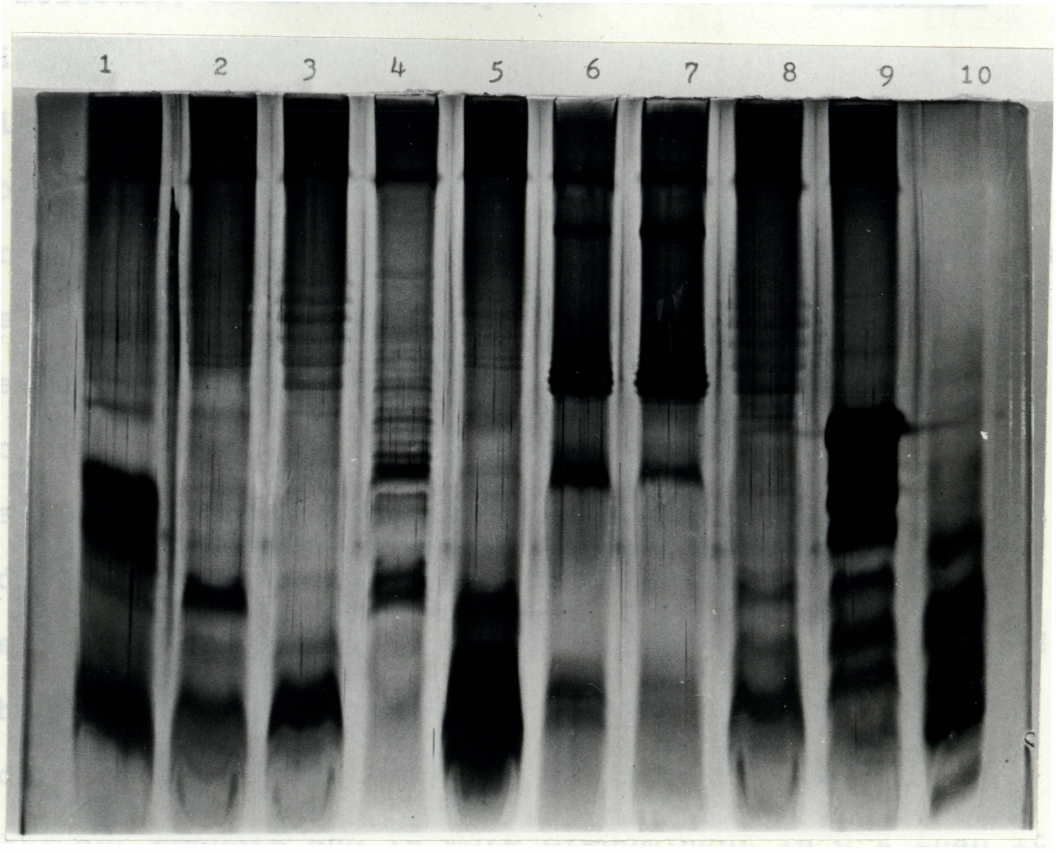

Figure 10b. SDS-PAGE of the material isolated in the phenol layer after phenol/water extraction of several strains of Rhizobium and Bradyrhizobium bacteria. Lane 1, R. leguminosarum ANU 54; lanes 2-8 are different strains of B. japonicum: 2, 61A76; 3, 61A101C; 4 and 5, USDA 110 and its mutant HS123 respectively; 6, 61A123; 7, 61A135; 8, ML144 mutant of 61A101C; 9, R. fredii USDA 205; 10, protein standards. Lanes 1-9 contain 30 uL of a 1 mg/mL solution. Lane 10 contains 2 ug of the two standards used (B-lactoglobulin and lysozyme).

pyruvate. Low levels of KDO (<1%) appear to be another characteristic of Bradyrhizobium japonicum strains studied so far (8,44, and this report) which explains the inability to purify the LPS by assaying for the presence of this molecule, as is routinely done for other Rhizobium strains (6). The major difference between LPS O-1 and LPS O-2 is observed in the glucose composition, O-1 contains approximately 3% more glucose than O-2. The fact that galactose was not detected in LPS O-2 may not be significant since O-1 contains only 0.5 ± 0.2% of this sugar. The difference in KDO is also very small. The fatty acid composition, Table 2, reported as relative percent, is characterized by the presence of straight-chain acids with 3-hydroxymyristic acid, lauric acid, oleic acid and two unidentified fatty acids as major components. Lauric acid is the most abundant in O-1 and oleic acid is the most abundant in O-2. Palmitic acid is also present in significant amounts and is more predominant in O-2 than it is in O-1.

Mild Acid Hydrolysis

Treatment of Bradyrhizobium japonicum 61A123 under normal conditins for mild acid hydrolysis (1% acetic acid at 100°C for 1 hour) did not separate the polysaccharide region from lipid-A, as observed for other Rhizobium LPSs (6,9,59). If the polysaccharide is separated from the lipid, SDS-PAGE exhibits no bands. Figure 11 shows that 1% acetic acid hydrolysis for 1 hour does not alter 61A123 LPS.

Table 2. Chemical Composition of the LPSs from *Bradyrhizobium japonicum* 61A123

Component*	LPS O-1	LPS O-2
Fucose	10.6±1.7	10.5±0.7
Mannose	3.2±0.2	3.2±0.2
Galactose	0.5±0.2	n.d.
Glucose	6.8±0.3	4.0±0.1
2-Amino-2,6-dideoxyhexose (1)	1.5±0.3	1.5±0.0
2-Amino-2,6-dideoxyhexose (2)	10.4±1.3	11.1±0.9
Uronic Acid	1.6±0.2	1.6±0.2
2-Keto-3-deoxyoctonate (KDO)	0.70±.02	0.98±.08
Pyruvate	n.d.	n.d.
Acyl groups	4.1±0.5	4.4±0.9
Fatty acids**		
Lauric C12:0	27.85	14.06
Myristic C14:0	8.10	6.60
3-Hydroxymyristic 3-OHC14:0	24.56	22.57
Palmitic C16:0	10.89	14.76
Unidentified FA1	6.58	5.56
Oleic C18:1 trans	10.63	20.83
Stearic C18:0	3.29	4.17
Unidentified FA2	8.10	11.46

* Except for fatty acids, these values are the percent of total mass given as the average of triplicate samples.

** Amount of fatty acids are reported as relative percent.

To determine the necessary conditions for hydrolysis, samples were removed at various time intervals and examined by SDS-PAGE. Our results suggest that is necessary to hydrolyze the samples for five hours to separate the polysaccharide from the lipid-A. Whether this increase in time affects the glycosyl composition of the LPS has not been determined.

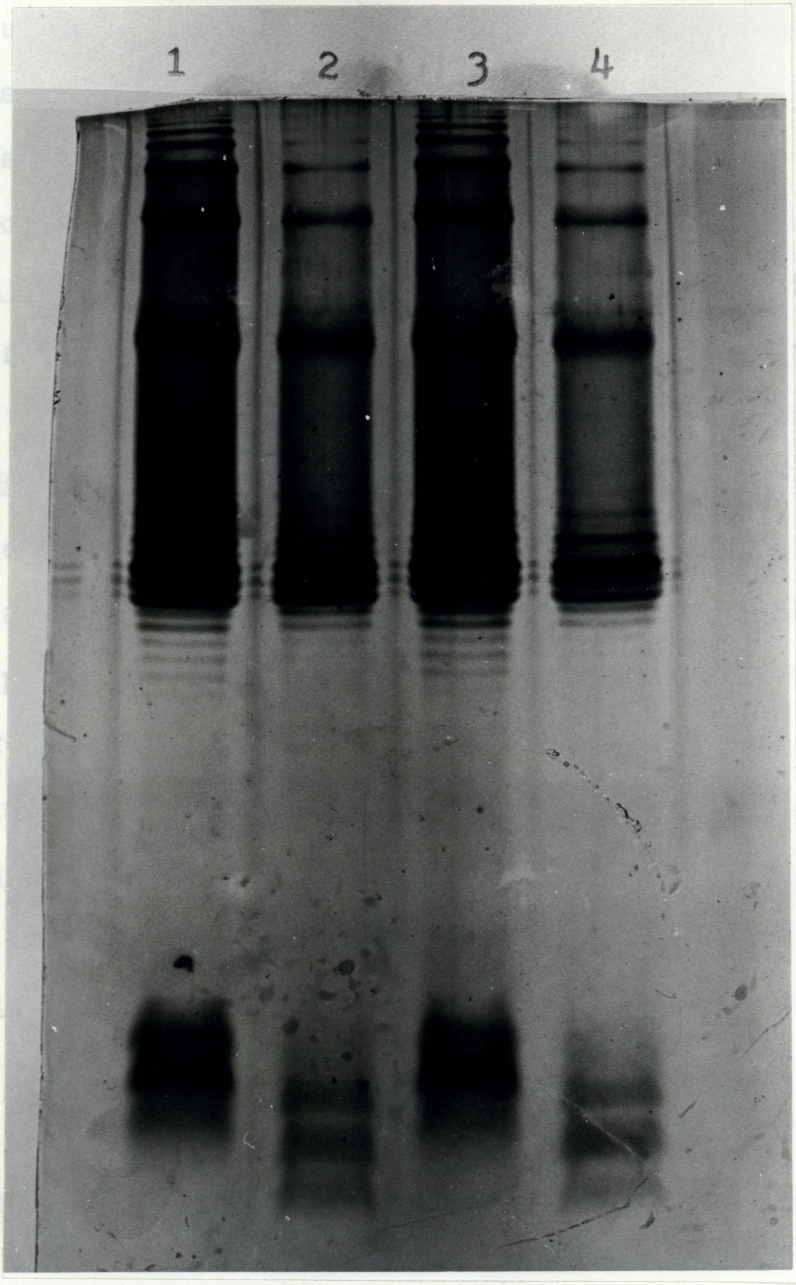

Figure 11. SDS-PAGE of B. japonicum 61A123 LPS O-1 (lanes 1 and 2) and LPS O-2 (lanes 3 and 4) after mild acid hydrolysis. Lanes 2 and 4 were hydrolyzed in 1% acetic acid for 1 hour at 100°C.

DISCUSSION

Results of this work confirm that the serogroup specific antigens isolated from Bradyrhizobium japonicum 61A123 are indeed lipopolysaccharides. However, these LPSs differ from those of other Gram-negative bacteria, including most Rhizobium species, in that they are isolated in the phenol phase of the phenol-water extraction (9,56). Previous reports had noted that the LPS from this strain and several other Bradyrhizobium japonicum strains were not found in the water layer (8). Recent studies of other Gram-negative bacteria have also reported the isolation of LPSs from the phenol phase (40), suggesting the presence of antigens that are more hydrophobic than normal lipopolysaccharides. Given these unusual characteristics, several other strains of Bradyrhizobium japonicum were studied to determine the nature of their LPSs. These strains are: Bradyrhizobium japonicum 61A76, 61A101 and mutants ML125, ML126 and ML144, and 61A135. Also, some strains with a known LPS composition were used for comparison. These are: Bradyrhizobium japonicum USDA 110 and its nonnodulating mutant, HS123 (44), Rhizobium fredii USDA205 (11) and Rhizobium leguminosarum ANU 54. These strains, with the exception of ANU 54, were grown in the new yeast extract-glutamate (YEG) medium, to facilitate the extraction of bacterial cells by the phenol-water method. ANU 54 was donated by a co-worker, Rick Scheuring.

Both layers of the phenol-water extraction were examined

by SDS-PAGE to determine the location of the LPSs. The material extracted into the phenol layer was incubated with proteinase K for one hour at 60°C prior to electrophoresis (24). Interestingly, all of the Bradyrhizobium japonicum strains contain LPSs that are isolated in the phenol phase (see Figure 10b). USDA 110 and its Nod$^-$ mutant appear to have an LPS that is partially extracted in both the phenol and the water layers (see Figure 10a). These results suggest that the possible presence of hydrophobic lipopolysaccharides (somewhat hydrophobic for USDA 110 and HS123) may be a general characteristic of the slow-growing Bradyrhizobium japonicum strains. At the present time there are no data to explain the nature of this hydrophobicity.

It is known that LPSs are the specific antigenic determinants in Rhizobium and Bradyrhizobium strains (9,59). Previous results have shown that antisera to 61A123 reacts strongly with 61A135 but not with USDA 110 (31). In the same way, phage experiments have demonstrated that the same type of phage will lyse 61A123 and 61A135 but not USDA110 (31). These results, together with the similarity observed in the gel pattern of B. japonicum 61A123 and 61A135 (Figure 10b), indicate that the LPSs of these two strains must have a comparable structure and chemical composition. Furthermore, since competition is determined by the different serogroups, and knowing that 61A123 and 61A135 have a similar receptor site for the interaction with phage (see above), it is likely that the structure of the

LPSs are related, directly or indirectly, to the process of competition.

Based on the ELISA results of B. japonicum 61A123 water soluble-phenol fraction after 24 hours of digestion with proteinase K, it is clear that this digestion step can be omitted since incubation alone, without PK, has similar effects on the release of antigenic material (see Figure 4).

The phenol-water extraction procedure (9,56) was chosen since it isolates the antigens in one fraction only, the phenol layer (Figure 3), as opposed to the other two methods in which the antigens were separated in two layers or membranes (Figures 6 and 7, respectively). In addition, this simple method can be completed in two days, whereas the other methods require about one week.

Results from the immunoblot indicate that antibodies to B. japonicum 61A123 are produced against the complete LPS only, as observed from the selective staining of the upper bands (Figure 8b). Isolation of the complete LPS could be accomplished by affinity chromatography. For this, the antibodies are purified by ion exchange chromatography (DEAE) and attached to another column (affinity column). After running the mixture of LPSs through this column, the incomplete LPS should pass through while the complete LPS would remain attached to the column. Elution of these molecules could be done by changing the pH or by a salt gradient.

This and purification of the polysaccharide region, after mild acid hydrolysis for five hours, are important

steps to further elucidate the structure of Bradyrhizobium japonicum 61A123 lipopolysaccharides.

Appendix I

A. Yeast Extract Mannitol Medium

Ingredients	Amount/Liter
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
Mannitol	5.0 g
Gluconic Acid	5.0 g
Yeast Extract	0.5 g
Agar(Difco), for solids	15.0 g

Adjust pH between 6.7-7.0 before autoclaving

B. New Yeast Extract Glutamate Medium

Ingredients	Amount/Liter
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
Glutamic Acid	7.0 g
Yeast Extract	0.5 g
Agar(Difco), for solids	15.0 g

Adjust pH between 6.7-7.0/NaOH before autoclaving

Appendix II

Modified LPS extraction (Hot phenol/water method)

Reagents:

A. Phosphate/EDTA buffer

0.05 M $Na_2HPO_4 \cdot 7H_2O$, 5 mM Na_2EDTA, 0.02% NaN_3, pH 7.0/HCl

B. Lysozyme

C. 90% phenol, liquid

D. 0.04 M $MgCl_2$

E. DNase I and RNase A

Procedure:

1. Put 150 mL of reagent A in a 400 mL beaker and set on ice
2. Weigh out bacteria pellet (20-30 g), transfer to beaker and stir to suspend.
3. Blend in Waring blender in the cold room, 3 x 1 min.
4. Sonicate (setting beaker on ice) using the Bronson sonic disruptor at 70% maximum power, 10 x 30 sec.
5. Add 15 mg of lysozyme, cover the beaker and stir overnight at 4°C.
6. Set up one water bath to 37°C and a second to 60°C. Heat the above mixture to 37°C and stir for 20 min. Aside, heat 200 mL of phenol to 65°C.
7. Repeat sonication procedure. Add 50 mL of reagent D, 10 mg of DNase and 100 mg of RNase. Incubate for 10 min at 37°C and then 10 min at 60°C.

8. Heat mixture to 65°C. Add preheated phenol and stir 15 min at 65°C. Cool on ice bath for 15 min and then centrifuge at 9,000 rpm for 20 min. Carefully remove water layer (top) and save.
9. Heat 200 mL of deionized water to 65°C and add it to the phenol layer; stir 15 min at 65°C. Cool on ice and centrifuge as before. Remove water layer and combine with first collection.
10. Dialyze and lyophilyze both the phenol and water layers.

Appendix III

Hexose assay

Reagents:

A. 0.2% Anthrone in conc. H_2SO_4

B. Standard: 1 mg/mL solution of glucose

C. Samples: 1 mg/mL solution

Procedure

1. Prepare a set of standards from 0 to 200 uL and q.s. to 500 uL with deionized water
2. Prepare a series of tubes for the samples but use larger volumes, 50-300 uL, and q.s. to 500 uL with deionized water
3. Add 1 mL of reagent A. Vortex and wait 5 min
4. Read absorbance at 620 nm

Appendix IV

KDO (2-keto-3-deoxyoctonic acid) Assay

Reagents:

A. 0.4 N H_2SO_4 (For strong acid hydrolysis use 10 N)

B. 0.04 N HIO_4 in 0.4 N H_2SO_4

C. 2% $NaAsO_2$ (sodium arsenite) in 0.5 N HCl

D. 0.3% thiobarbituric acid

Procedure

The nature of samples determines the amount needed. All samples are diluted to a final volume of 200 uL.

1. Prepare standards from a 0.1 mg/mL solution of KDO. Dilute to 200 mL with deionized water.
2. Add 20 uL of reagent A to standards and samples, vortex.
3. Place in boiling water bath for 30 min (for strong acid hydrolysis, leave 1 hr).
4. Add 250 uL of reagent B, vortex and leave at room temperature for at least 40 min.
5. Add 500 uL of reagent C, vortex and wait for 5 min.
6. Add 2 mL of reagent D, vortex and place in boiling water bath for 20 min.
7. Read absorbance immediately at 548 nm. Centrifuge any cloudy samples for 1 min.

Appendix V

ELISA (Enzyme Linked Immunosorbant Assay)

Reagents

A. PBS-Tween Buffer

0.137 M NaCl, 1.47 mM KH_2PO_4, 2.68 mM KCl, 8.38 mM $Na_2HPO_4 \cdot 12H_2O$, 0.02% NaN_3, 0.5 mL Tween 20, final pH 7.4

B. Coating Buffer

15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6

C. Substrate Buffer

0.1 M Glycine, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $ZnCl_2$, pH 10.5/NaOH

D. Substrate: p-nitrophenyl phosphate disodium

E. Rabbit anti-Rhizobium antiserum

F. Conjugate: Goat anti-rabbit IgG alkaline phosphatase

G. 0.85% NaCl

H. 3 M NaOH

Procedure

1. Wash bacteria (antigen) from slant using minimum amount of reagent G (about 1 mL)
2. Heat in boiling water bath for 15 min; allow to cool
3. Dilute with reagent B to an optical density (O.D.) of 0.5 absorbance at 620 nm
4. Coat the wells of a microtiter plate with 100 uL of the antigen-coating buffer suspension
5. Cover the plate and allow to incubate at $5^{o}C$ overnight or $37^{o}C$ for 1 hr (coating incubation)

6. Once coating is complete, shake excess antigen out of wells and wash plate with reagent A four times. During first washing, the plate was filled with PBS-Tween and immediately emptied. For the following washes PBS-Tween remained in the wells for 5 min before removal
7. To positive control wells add 50 uL of a 1:400 dilution of reagent E in PBS-Tween, and 50 uL of reagent A for a final dilution of 1:800. To negative control wells add 100 uL of reagent A only. To experimental wells add 50 uL of the 1:400 dilution of reagent E in PBS-Tween, and 50 uL of serial 2-fold dilutions of sample, in PBS-Tween. Cover the plate and allow to incubate at 28^{o}C for 1 hour (anti-Rhizobium incubation)
8. Repeat washing procedure
9. To each well add 100 uL of a 1:1000 dilution of reagent F in PBS-Tween, and allow to incubate at 5^{o}C overnight or 37^{o}C for 3 hrs (conjugate incubation)
10. Repeat washing procedure
11. Add 100 uL of a substrate solution, 1 mg/mL of reagent C, to each well. Allow to develop at room temperature (substrate incubation). After the yellow coloration of positive wells is apparent, 20-30 min, stop the reaction by adding 50 uL of 3 M NaOH per well
12. Read absorbance at 405 nm

Appendix VI

Discontinuous SDS-Polyacrylamide Gel Electrophoresis

Stock solutions:

A. Running gel stock buffer

1.875 M Tris base, pH 8.8/HCl

B. Stacking gel stock buffer

0.635 M Tris base, pH 6.8/HCl

C. Stock 50% acrylamide solution

25 g Acrylamide, 0.625 g Bis-acrylamide. Add deionized water to a total volume of 50 mL

D. Sodium dodecyl sulfate (SDS) solution, 10%

2.5 g SDS in 25 mL Deionized water

E. Sample buffer

5 mL Solution B, 2 g Sucrose, 0.4 g SDS, 5 mg Bromphenol blue, 1 mL B-mercaptoethanol. Dilute to 20 mL with deionized water

F. Running buffer

0.0248 M Tris base, 0.192 M Glycine, 3.47 mM SDS

Preparing the running gel

1. Assemble the gel electrophoresis apparatus.
2. For 30 mL of a 15% running gel, mix the following:

   6.0 mL solution A, 9.0 mL solution C, 0.3 mL solution D, 14.7 mL deionized water, sucrose (optional) 10% w/v
3. Just before pouring the gel add 0.07 mL of a freshly prepared 10% ammonium persulfate solution (in degased deionized water) and 0.03 mL of TEMED (N,N,N',N'-Tetramethylethylenediamine) to the gel solution and mix.

4. Pour the gel using a Pasteur pipette. Avoid bubbles.
5. Using a glass syringe carefully cover the surface of the gel with 1.5 mL of deionized water. Alternative: Use a running gel solution without acrylamide or sucrose.
6. Allow to polymerize, preferably overnight.

Preparing the stacking gel

1. For 10 mL of 5% stacking gel, mix the following: 2.0 mL solution B, 1.0 mL solution C, 0.1 mL solution D, 6.9 mL deionized water
2. Remove the water overlaying the running gel and blot dry with a paper towel.
3. Just before pouring the stacking gel, add 0.05 mL of a freshly prepared 10% ammonium persulfate solution and 0.02 mL of TEMED and mix.
4. Pour the stacking gel using a Pasteur pipette. Avoid bubbles.
5. Slowly insert the well former to about 1 cm from the running gel and do so at a slight angle to avoid trapping air bubbles beneath the wells. Allow to polymerize.

Preparing the samples

1. To lyophilized samples add 25 uL of solution E and boil in a water bath for 5 min.
2. Fill the upper gel reservoir with running buffer (solution F) and carefully remove the well former.
3. Load one sample in each well. Fill the lower reservoir with running buffer and assemble electrical connections.

4. Run at constant current of 20 ma. Electrophoresis is complete when the bromphenol blue reaches the bottom of the gel (4-5 hrs).
5. Turn off the power, empty reservoirs and carefully disassemble apparatus to remove the gel.
6. Stain the gel for appropriate macromolecules.

Appendix VII

Cell Membrane Isolation

Reagents:

A. Buffer A

0.02 M Tris, 5 mM EDTA, 0.25 M sucrose, pH 7.8

B. Buffer B

0.02 M Tris, 0.05 M $MgCl_2$, pH 7.8

C. Lysozyme

D. DNase I

Procedure:

1. Freeze pellet in liquid N_2 and grind into a powder
2. Suspend in buffer A (powder should account for 30% of final volume) and add 0.5 mg of lysozyme per mL.
3. Freeze by swirling in liquid N_2
4. Thaw in warm water until just melted and pour into 20 volumes of cold buffer B, containing 0.1 mg of DNase per mL of solution
5. Blend immediately for 20 sec to shear DNA and disperse the cells
6. Centrifuge at 5,000 x g for 5 min to remove unbroken cells. If pellet is substantial, it can be suspended again in buffer A and the cycle (steps 3-6) is repeated
7. The envelope material, consisting of inner and outer membranes, is recovered by centrifugation at 27,000 x g for 20 min. Dialyze and lyophilize.

Appendix VIII

Isolation of Outer and Inner Membranes

Reagents:

A. 5 mM EDTA, pH 7.5

B. Suspension Buffer

3.3 mM Tris, 5 mM EDTA, 25% sucrose (w/w), pH 7.5

C. Sucrose solutions (w/w):

35%, 40%, 45%, 50%, 55% in 5 mM EDTA, pH 7.5

Procedure:

1. Prepare sucrose gradient using 3 mL of each solution (reagent C) per centrifuge tube
2. Suspend cell envelope in 1 mL of reagent B and overlay on top of sucrose gradient
3. Centrifuge at 82,000 x g, 2-4^{o}C, for 36-48 hrs
4. Collect bands, outer membrane bottom, inner membrane top, using a Pasteur pipette. Dialyze in 12,000-14,000 Molecular Weight cutoff tubing, and lyophilize.

Appendix IX

Uronic Acid Assay

Reagents

A. 0.0125 M Sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) in conc. H_2SO_4. Stored at 4^oC

B. 0.5% NaOH

C. 0.15% m-hydroxybiphenyl in 0.5% NaOH. Stored at 4^oC

D. Standard glucuronic or galacturonic acid (0.1 mg/mL)

Procedure

1. Prepare a set of standards 0-200 uL and q.s. to 200 uL with deionized water
2. Prepare two sets of samples with a total volume of 200 uL
3. Add 1.2 mL reagent A. Vortex
4. Heat for 5 min in a boiling water bath
5. Cool 1-2 min in cold water
6. To the standards and one set of samples add 20 uL of reagent C. To the other set of samples add 20 uL of reagent B. Vortex
7. Leave at room temperature for 5 min
8. Read absorbance at 520 nm. Subtract any reading of the samples treated with NaOH from those treated with m-hydroxybiphenyl for the true reading

Appendix X

Assay for Acetyl groups

Reagents

A. 2 M Hydroxylaminehydrochloride. Stored at $4^{o}C$.

B. 3.5 M NaOH

C. 0.37 M $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl

D. HCl, 1 part conc. HCl + 2 parts H_2O

E. Standards: 0.5 mg/ml B-D(+)-glucose pentacetate in CH_3OH. Stored at $4^{o}C$. 10 uL= 2.75 ug acetate.

Procedure

1. Lyophilize 400-600 microliters of a 1 mg/mL solution of samples
2. Pipette different volumes of standard (recomended: 0, 10, 20, 40, 80, 100 microliters), add 300 microliters of H_2O to each and dilute to a final volume of 400 microliters with CH_3OH
3. To 200 microliters of sample, add 100 microliters of CH_3OH and 100 microliters of H_2O. To the lyophilized samples add 100 microliters of CH_3OH and 300 microliters of H_2O
4. Mix equal parts of reagents A and B just before using. This is stable for three hours at room temperature.
5. Add 800 microliters of the mixture to samples and standards. Vortex and allow to stand for 1 min.
6. Add 400 microliters of reagent D (HCl). Vortex to mix
7. Add 400 microliters of reagent C ($FeCl_3$). Vortex
8. Measure absorbance at 540 nm.

Note: If samples contain significant quantities of protein, this will precipitate out upon addition of reagent C. Centrifuge

Appendix XI

Pyruvic Acid Assay

Reagents

A. 2,4-dinitrophenylhydrazine (DNP). 500 uM in 100 mL of 2.0 N HCl. Freshly prepared. Dissolve at 40°C

B. 2.0 N HCl

C. Toluene

D. 10% Na_2CO_3

E. 2.2 N NaOH

F. Standard (0.1 mg/mL). Freshly prepared

Procedure

1. Prepare a set of standards 0-200 uL and q.s. to 200 uL with deionized H_2O. Use screw cap test tubes
2. Prepare samples from 200 to 400 ug in screw cap test tubes and add 200 uL of H_2O. If samples contain protein treat with perchloric acid
3. Add 300 uL of reagent B, vortex, cap the tubes and heat at 100°C in heating block for 3 hrs
4. Add 100 uL of A and set at room temperature for 30 min
5. Add 600 uL of reagent C and vortex. Remove the aqueous phase using a Pasteur pipette. To the toluene layer add 600 uL of reagent D and vortex
6. Discard toluene layer (top). Add 400 uL of H_2O and 1.0 mL of reagent E to the bottom layer. Vortex
7. Centrifuge for 1 min and read absorbance at 416 nm

Appendix XII

Preparing Alditol Acetates

Reagents:

A. Inositol (1 mg/mL solution), as internal standard

B. Standard sugars (1 mg/mL solution): rhamnose, fucose, ribose, xylose, mannose, galactose, glucose, and heptose

C. 2.0 M trifluoroacetic acid (TFA)

D. Sodium borohydride (or sodium borodeuteride): 10 mg/mL solution in 1 M ammonium hydroxide

E. Glacial acetic acid

F. 10% glacial acetic acid in methanol

G. Methanol

H. Pyridine

I. Acetic anhydride

J. Chloroform

Procedure:

1. Determine the percent hexose in the sample using the anthrone assay
2. Place sample equivalent to not more than 250 ug hexose in screw-cap test tube. In another tube, place 100 uL of each standard. Add 20 uL of inositol to sample and standards. Dry materials by blowing filtered air or by freeze-drying
3. Add 500 uL of TFA to each tube, seal with teflon-lined screw cap, and heat at 121^{o}C for 2 hrs
4. Remove tubes and blow dry with filtered air. This may be done in a water bath at 45^{o}C. For methylated

polysaccharides, keep the temperature under 35°C

5. Add 250 uL of sodium borohydride solution, mix and allow to stand at room temperature for 1 hr (can be left overnight with loose caps on). For methylated samples always use sodium borodeuteride, and leave at room temperature for 2 hrs
6. Add 50 uL of glacial acetic acid. Vigorous bubbling will take place. Repeat two more times
7. Add 500 uL of the 10% glacial acetic acid in methanol and blow dry with filtered air. Keep drying temperatures as mentioned previously. Do this four times
8. Add 500 uL of methanol and blow dry as in step 7
9. Add 50 uL of pyridine and 50 uL of acetic anhydride. Mix, seal tubes with teflon caps and heat at 121°C for 30 min
10. Cool on ice and extract by adding 500 uL of water and 500 uL of chloroform. Mix, centrifuge for 5 min on clinical centrifuge, remove chloroform layer (bottom) and transfer to another tube
11. Extract water layer with another 500 uL of chloroform and combine chloroform layers. Blow dry with filtered air. Analyze by gas chromatography

Appendix XIII

Fatty Acids Analysis

Reagents:

A. 4 N HCl

B. 4 N NaOH

C. 6 N HCl

D. Petroleum ether

E. Boron trifluoride methanol complex (hood)

F. Internal standard: lauric acid 1 mg/mL in chloroform (stored at $4^{o}C$)

G. Samples to be analyzed: 1 mg/mL solution of LPS

H. Standards: 10 uL of each (freezer)

Procedure:

1. In a set of screw cap test tubes place 100 uL lauric acid as internal standard. Add 500 uL of sample to the first tube, and 10 uL of each standard to the second tube. Lyophilize
2. Add 500 uL of reagent A and heat for 2 hrs at $100^{o}C$, to hydrolyze. (It is possible during hydrolysis for two fatty acids to join together through oxygen, i.e., -C-O-C-, therefore, it is necessary to perform a second, alkaline, hydrolysis)
3. Add 1.0 mL of reagent B, vortex and heat for 2 hrs. at $100^{o}C$ in heating block, to hydrolyze. The product of alkaline hydrolysis is the salt of the fatty acids.
4. Acidify with 0.5 mL of reagent C to obtain fatty acids; vortex. (Now you have the fatty acids but also other

substances that make up the LPS)

5. Extract fatty acids with 1.0 mL of reagent D. Remove top layer, BEING CAREFUL NOT TO PIPETTE BOTTOM LAYER, and transfer to clean screw cap test tubes which are in a rack set in ice. Repeat extraction 2 more times. Blow dry while tubes are in ice
6. Add 500 uL of reagent E while tubes are cool and heat for 2 min in heating block at 100^{o}C (esterification step)
7. IMMEDIATELY put tubes back in ice, allow to cool, and add 1.0 mL deionized water; vortex to mix (if water is not added, the boron trifluoride is not dissolved and goes into the petroleum ether layer in next step)
8. Extract esters with 1.0 mL of reagent D. Remove top layer, AVOIDING ANY WATER, and transfer to clean test tubes set in ice. Repeat extraction 2 more times. Blow dry samples while in ice
9. If you are going to estimate esters on the GC immediately, keep blow-dried samples in ice, otherwise store in the cold room

NOTE: GC parameters are: 150/4/4-250/10 corresponding to temp, init. time, rate/min, final temp, and final time respectively.

Appendix XIV

LPS Extraction by EDTA/$CHCl_3$/CH_3OH method

Reagents:

A. PBS buffer

0.05 M $Na_2HPO_4 \cdot 7H_2O$, 0.145 M NaCl, pH 7.4/HCl

B. 0.01 M EDTA in PBS buffer pH 7.4

C. Chloroform

D. Methanol

Procedure:

EDTA Extraction

1. Wash cell pellet with PBS 3 times to remove medium contaminants
2. Suspend cells in reagent B (1 g wet cells/10 mL buffer)
3. Shake for 1 hour at 45°C
4. Centrifuge at 12,000 x g for 20 min
5. Dialyze supernatant (48 hrs, 6 x 20 L)
6. Centrifuge as above and filter through 0.22 um pore size
7. Lyophilize

Folch extraction

8. Dissolve EDTA extract in PBS pH 7.4 (25 mg/mL buffer)
9. Add 75 mL $CHCl_3$:CH_3OH (2:1). Stir 1 hr at room temp.
10. Filter mixture through sintered glass
11. Wash insoluble material with few mL of $CHCl_3$:CH_3OH
12. To filtrate add $CHCL_3$:CH_3OH (2:1) to a final volume of 80 mL
13. Extract with 16 ml of PBS
14. Store mixture at -20°C overnight

15. Centrifuge at 10,000 x g for 1 hr and separate phases
16. Reextract upper layer with 40 mL of theoretical lower layer ($CHCl_3$-MeOH-PBS, 86:14:1), and lower layer with theoretical upper layer ($CHCl_3$-MeOH-PBS, 3:48:47)
17. Separate phases as before
18. Concentrate layers and dialyze for 48 hrs
19. Lyophilize

Appendix XV

Silver Staining for LPSs

Reagents:

A. Fixing solution

25% isopropanol, 10% glacial HAc, 65% deionized water

B. Oxidation solution (prepare just before use)

1.05 g $NaIO_4$, 150 mL deionized water, 4 mL of reag. A

C. Silver stain (prepare just before use and stir constantly while making)

115 mL deionized water, 28 mL 0.1 M NaOH, 5 mL 20% $AgNO_3$, 1 mL NH_4OH. Brown precipitate should disappear with NH_4OH. If not add more drop-wise

D. Developer solution (prepare just before use)

0.05 g citric acid, 0.5 mL of 37% formaldehyde, 0.5 L to 1 L of deionized water

Procedure:

1. Fix the gel overnight in reagent A
2. Oxidize for 5 min in reagent B with shaking
3. Wash 3 x 15 min with deionized water by shaking
4. Silver stain for 10 min with shaking
5. Repeat wash
6. Develop the bands to desired darkness in reagent D
7. Place gel in stop bath (optional) consisting of 200 mL of deionized water plus 10 mL of 7% (v/v) acetic acid;
8. Wash 3 x 10 min with deionized water and store

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